Sodium Fluxes in Desheathed Frog Sciatic Nerve

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ABSTRACT Desheathed frog (*R. pipiens*) sciatic nerves were soaked in solutions that contained ouabain or NaN_3, in K-free solutions, or in K- and Ca-free solutions. Then, the nerves were allowed to recover in standard Ringer's solution. At various times during the soaking periods, some nerves were analyzed for Na and K, and estimates were made of the influx and efflux of Na^{22}. While a nerve was soaking in any one of the experimental solutions, the Na^{22} influx was increased, the Na content was rising, and the K content was falling by an equivalent amount. The rate coefficient for Na^{22} efflux was reduced by about 25 per cent by 0.05 mM ouabain and by about 50 per cent by 5 mM NaN_3. Potassium-free solutions had little effect on the rate coefficient. It was concluded that the efflux of Na from frog nerve is dependent on the metabolism, but not on the external concentration of K. The equimolar exchange that is characteristic of the net movements of Na and K in frog nerve may not be due to the presence of a tightly coupled Na–K exchange pump, but may represent a constraint imposed by the requirement for electroneutrality.

INTRODUCTION

The efflux of radioactive Na from the giant axons of cephalopods is dependent on the cellular metabolism and on the concentration of K in the bathing solution (5). Similar results have been obtained by workers studying other types of cells, and several workers have suggested that cellular membranes contain an ion pump that is driven by the metabolism and that carries Na out of a cell and transports K into a cell (3-5, 10, and 20). However, Shanes (16) has reported that, under some conditions, the efflux of Na from desheathed toad sciatic nerves is not reduced when K is omitted from the bathing solution, and he has proposed a different mechanism for cation transport in toad nerves (17). It seemed worth while to document further this difference between the giant axons of cephalopods and the peripheral nerve fibers of frogs and toads. Measurements were made of the Na and K contents of desheathed frog sciatic nerves and of the rates of Na^{22} exchange when the nerves were soaked in various modifications of the standard Ringer's solu-

tion. The results are similar to those reported by Shanes (16), and they indicate that the sodium transport system in frog nerve differs from that in giant axons in two respects: namely, the Na efflux from frog nerve is not very sensitive to ouabain or to the removal of K from the external solution.

MATERIALS AND METHODS

General Desheathed sciatic nerves from the frog *Rana pipiens* were used throughout the investigation. Usually each nerve was soaked in a vial that contained approximately 10 ml of a solution that was equilibrated with a gas mixture composed of 78 per cent N_2, 20 per cent O_2, and 2 per cent CO_2. When the Na^{22} influx was measured, however, as many as four nerves were placed for 0.5 hour in 5 to 6 ml of radioactive solution. Dry weights were determined after the nerves had been dried beneath an infrared lamp for at least 0.75 hour, or after they had been dried overnight in an oven at 70–80°C. The results of all analyses were referred to the dry weight. The numerical values quoted in the text represent averages ± 1 SD. Unless indicated otherwise, all experiments were carried out at 20°C.

Solutions The standard Ringer's solution contained 111 mM NaCl, 2.0 mM KCl, and 1.8 mM $CaCl_2$. In the influx experiments it contained, in addition, 4.8 mM $NaHCO_3$ and was buffered to pH 7 by equilibration with the gas mixture described above. In the efflux experiments, the solutions were buffered with 2 mM Na_2HPO_4 and 1 mM NaH_2PO_4 and equilibrated with air. When changes in the composition of the bathing solution were made, the NaCl was adjusted to maintain the tonicity constant.

Preparation of Radioisotope Carrier-free Na^{22} Cl in HCl was diluted with 120 mM NaCl to a concentration of about 60 μc/ml, and the acidity was adjusted to pH 6–8 with NaOH. Aliquots of this stock solution were added to the various experimental solutions to make them radioactive.

Extraction and Analysis When the uptake of Na^{22} and the Na and K contents of a nerve were to be measured, the nerve was washed for 0.5 hour at 2°C in a solution that contained 118 mM choline chloride and 1.8 mM $CaCl_2$. This was done to reduce the quantity of Na and K contained in the extracellular spaces. Hereafter, this washing solution will be referred to as solution W. After the nerve had been washed, 4 to 5 mm were cut from each end and the central portion was dried, weighed, and extracted by leaching in deionized water at room temperature for 2 to 5 days (6). The aqueous extract was analyzed for Na and K with a flame spectrophotometer, and an aliquot of the extract was counted.

In the efflux experiments, the nerves were not washed in solution W, and they were extracted in 1.0 ml of water at 100°C for 15 minutes. By counting some of the dried nerves before and after extraction, it was found that this procedure removed 85 to 95 per cent of the radioisotope, and an average of 90 per cent was assumed in analyzing the data (this was checked for Na^{22}, Rb^{86}, and Cs^{134}).

For the Cl determinations, dried nerves were extracted for several days in the refrigerator in 1.0 ml of 0.4 N H_2SO_4, and the extracts were analyzed with an Aminco-Cotlove automatic chloride titrator.

Effects of Cut Ends To minimize any effects produced by damaged tissue, 4 to 5 mm were cut from the ends of the nerves that were used in the influx experiments. In the efflux experiments, however, any leakage from the cut ends would contribute to the loss of radioisotope. To estimate the magnitude of this source of error, nerves were cut into three pieces at the end of the efflux run, and the end portions were counted separately from the central portion. In two of three cases, the Na^{22} contents (CPM/gm) of the end sections were 10 to 20 per cent lower than for the central section, and in a third nerve about 20 per cent higher. It seems unlikely that diffusion from the ends accounts for a large portion of the efflux of radioisotope.

Flux Determinations If a frog nerve contains a single well stirred compartment, the axoplasm, the differential equation that describes the movements of Na^{22} between the axoplasm and the bathing solution is:

$$\frac{dq_i^*}{dt} = \frac{M_i c_o^*}{c_o} - \frac{M_o q_i^*}{Q_i} \qquad (1)$$

where: t = the time in hours
q_i^* = the quantity of Na^{22} in the nerve in CPM/gm (dry)
c_o^* = the concentration of Na^{22} in the external solution in CPM/ml
c_o = the concentration of Na in external solution in μmols/ml
M_i = the Na influx into the axoplasm in μmols/(gm $\times$ hr.)
Q_i = the quantity of Na in the nerve in μmols/gm (dry)
M_o = the Na efflux from the axoplasm in μmols/(gm $\times$ hr.)

If the axoplasm is the only compartment in a nerve, then the influx of Na into the axoplasm can be estimated from the amount of radioactivity taken up by the nerve during a brief exposure to a solution that contains Na^{22}. (The concentration of Na^{22} was about 1 μc/ml.) If the duration of the exposure to the radioactive solution is small, then the second term on the right of equation (1) may be neglected and the influx calculated from the formula:

$$M_i = \frac{1}{t}\left[\frac{q_i^* c_o}{c_i^*}\right] \qquad (2)$$

If a nerve contains compartments other than the axoplasm, then the Na^{22} contents of the extraneous compartments must be deducted from the total Na^{22} content of the nerve before equation (2) is used.

For equation (2) to be valid, t should be small compared to the time constant, $\tau = Q_i/M_o$, for the exchange of radioisotope in a nerve in a steady state. For Na^{22}, τ is approximately 2 hours, and t was chosen to be 0.5 hour. In this paper, the Na^{22} content of a nerve is expressed in μmols/gm. To compute this quantity for a given nerve, the radioactivity in the nerve (CPM/gm) was divided by the specific activity of the bathing solution. This is the quantity in brackets in equation (2). It is the Na^{22} content that would have been attained if all the Na in the bathing solution had been replaced with Na^{22}.

For the efflux experiments, the nerves were equilibrated for 3 to 5 hours in 2 to 3 ml of a solution that contained Na^{22} at a concentration of 5 to 10 μc/ml. Each nerve

was then passed through a series of planchets, each of which contained approximately 1.5 ml of inactive solution. A nerve was lifted by a blunt glass hook to transfer it between successive planchets. Usually, the time spent in the first four planchets was 2, 3, 5, and 10 minutes, respectively; the nerve remained 20 minutes in each of the subsequent planchets. After removal from the last planchet, the nerve was dried, weighed, and rapidly extracted. An aliquot of the extract was pipetted into a planchet containing 1.5 ml of solution, and all planchets were dried and counted.

In an efflux experiment, $c_o^* = 0$, and:

$$\frac{dq_i^*}{dt} = - M_o \, q_i^*/Q_i$$

Rearranging:

$$\frac{M_o}{Q_i} = \frac{-1}{q_i^*} \frac{dq_i^*}{dt} \approx \frac{1}{\bar{q}_i^*} \frac{(-\Delta q_i^*)}{\Delta t} \equiv k \qquad (3)$$

where: $(-\Delta q_i^*)$ = Na^{22} in a given planchet
Δt = time (hours) spent in that planchet
$\bar{q}_i^*$ = mean quantity of Na^{22} in the nerve while it was in that planchet

The quantity, k, hereafter referred to as the rate coefficient for Na^{22} efflux, is a measure of the ratio of the Na efflux to the Na content of a nerve (M_o/Q_i), if the nerve consists of a single well stirred compartment. If a nerve consists of several compartments with unequal rates of Na^{22} exchange, then k will continually decrease during an efflux experiment and approach the value of the rate coefficient of the slowest exchanging compartment in the nerve. At these late times, k will be a measure of the ratio of the Na efflux to the Na content of the slowest exchanging compartment. If this compartment is in a steady state, k will eventually become constant; if the Na efflux is proportional to the Na content, then k will approach a constant even in a non-steady state. In any event, if the Na efflux from this slowest compartment changes, then for times so short that the Na content of the compartment does not change appreciably, the changes in k will be proportional to the changes in the efflux.

When solutions were changed during an efflux experiment, a check was made to determine whether the dried residues of different solutions absorbed equally the radiation from Na^{22}. When a given quantity of Na^{22} was counted in the dried residue from isotonic solutions of sucrose or choline chloride, the counting rate was only half that obtained when the counting was carried out in the residue from Ringer's solution.

RESULTS

Compartments in Nerve When the perineurium is removed from a frog or bullfrog sciatic nerve, the nerve swells and takes up Ringer's solution (6, 15). After a few hours, approximately 90 per cent of the Na in the nerve resides in the extracellular space. Previous work (6) had indicated that washing a frog nerve for 0.5 to 1 hour in a cold solution of 118 mM choline chloride

and 1.8 mM $CaCl_2$ (solution W) removed nearly all of the Na from the extracellular spaces and caused only a small loss of Na from the cells. A washout procedure was employed in this investigation to eliminate most of the Na and Na^{22} from the extracellular spaces of the nerves before they were prepared for analysis. Since a rather small quantity of Na^{22} penetrates into the intra-

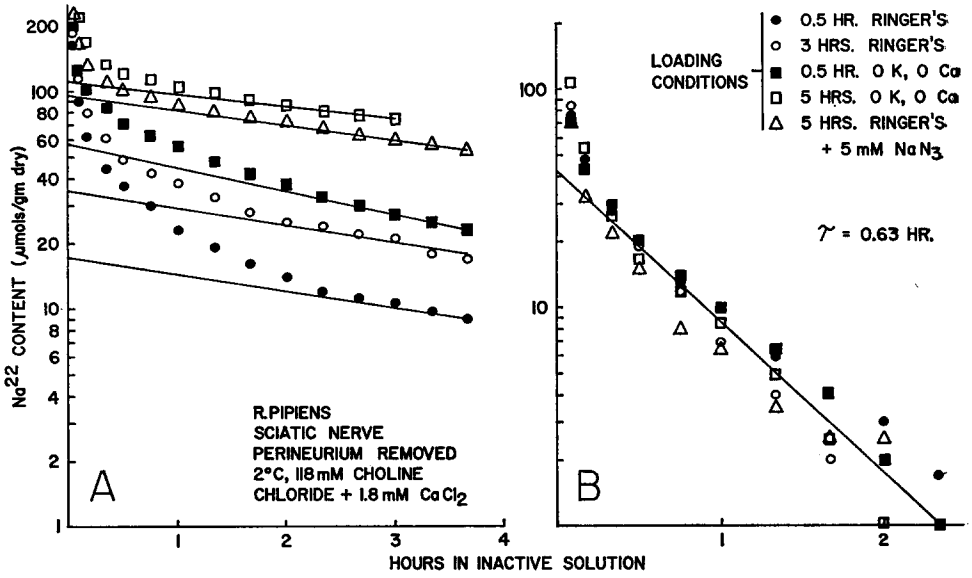

FIGURE 1A. Time course of the decline of the Na^{22} contents of nerves loaded at 20°C under various conditions and then washed at 2°C in solution W. Ordinate, Na^{22} content on a logarithmic scale. Abscissa, time after beginning the washout of Na^{22}. The Na^{22} content was obtained by dividing the radioactivity in the nerve (CPM/gm) by the specific activity of the loading solution. The straight lines were fitted visually to the last several points and define the "intracellular" component of Na^{22}. Each curve is an average of from two to four experiments.

FIGURE 1B. Average values of the differences between the total Na^{22} contents and the intracellular components defined in Fig. 1A. The differences were determined separately for each nerve and not from average curves shown in Fig. 1A. Ordinate and abscissa as in Fig. 1A. The straight line was fitted visually to the data and defines the intermediate component.

cellular space of a nerve that has been soaked for 0.5 hour in a radioactive solution, it is important to have a fairly accurate estimate of the quantity of extracellular Na^{22} that remains in a nerve trunk after it has been washed in cold solution W for 0.5 to 1 hour. To obtain this information, nerves were loaded with Na^{22} under the various conditions indicated in Fig. 1, and the Na^{22} was then washed out at 2°C in solution W. The time courses of the declines in the Na^{22} contents of these nerves (desaturation curves) are shown in Fig. 1A.

Before these nerves were placed in the first planchet, they were blotted a few times on filter paper, but no attempt was made to remove superficial radioactivity by preliminary dips in non-radioactive solutions. The initial values of the Na^{22} contents of the nerves are not shown in Fig. 1. They ranged between 400 and 600 μmols/gm, which correspond to Na spaces of 3.3 to 5.0 ml/gm. The first few points of the curves in Fig. 1 may be inaccurate because preliminary dips were not used, but this is not considered serious for two reasons. First, none of the arguments presented here are based on the values for the total Na space. Second, the main difficulties encountered with extracellular Na were caused, not by a superficial component, but by a component which diffused slowly from the nerve. It should be noted also that the Na^{22} contents of the nerves have not been expressed as a percentage of the total initial Na^{22} content. Therefore, the presence of considerable quantities of superficial Na^{22} in the first few washout planchets did not affect the values calculated for the Na^{22} content of a nerve when it was in subsequent planchets.

Shanes's observations (15) show that the swelling of the desheathed bullfrog nerve is about 50 per cent completed after 20 minutes and about 80 per cent completed after 2 hours. In this investigation, all nerves were soaked for at least 2 hours after they had been desheathed before an efflux or an influx measurement was made. The stability of the total Na^{22} space was not investigated, but the results in Fig. 1 indicate that the slowly diffusing component of the extracellular Na^{22} was quite constant.

The curves in Fig. 1A contain at least three components. One component is large, exchanges within a few minutes, and probably represents superficial Na^{22} and Na^{22} in the interstitial spaces of the nerve. The straight lines drawn through the last several points on these curves delineate slow components that are assumed to represent intracellular Na^{22}. The differences between these ruled lines and the original data are plotted in Fig. 1B. These curves reveal a third, or intermediate, component of the original desaturation curves that is not affected by the method of loading, contains 40 μmols Na^{22}/gm, and exchanges with a time constant of 0.6 hour. Because this component is not affected by conditions that produce large alterations in the ionic contents of a nerve, it is considered not to be intracellular Na^{22}. Because of its relatively slow exchange rate, it seems likely that this intermediate component is derived from a relatively inaccessible compartment of the extracellular space. It may represent Na^{22} in the myelin sheaths (18, 19), or it may represent Na^{22} associated with the fiber in some other manner. Müller's measurements (12) indicate that considerable Na may be associated with a single fiber.[1]

[1] The curves in Fig. 1B indicate that the size of the intermediate compartment is the same for a 0.5 hour exposure to a radioactive solution as for a 5 hour exposure. This seems inconsistent with the fact that this compartment exchanges with a half-time of about 0.5 hour at 2°C. The washout ex-

Huxley (8) has pointed out that, in general, it is incorrect to identify the intercepts of curves, such as those in Fig. 1, with the sizes of the various compartments in a piece of tissue. This identification overestimates the sizes of the slowly exchanging compartments. In the desheathed frog sciatic nerve, the time constants for the loss of Na^{22} from the interstitial space, the intermediate compartment, and the intracellular space are 2 minutes (6), 38 minutes, and 100 minutes, respectively. The relative sizes of these compartments are 8:1:1 (6). If the intermediate and intracellular compartments are assumed to be arranged in parallel with one another, but in series with the interstitial space, then, using Huxley's formula, one calculates that the intercepts in Fig. 1 overestimate the size of the intracellular compartment by approximately 2 per cent and overestimate the size of the intermediate compartment by approximately 5 per cent. The fact that the apparent size of the intracellular compartment can be changed without influencing the apparent size of the intermediate compartment indicates that it is correct to treat these compartments as being in parallel.

The existence of a slowly exchanging compartment in the extracellular space is also evident in Na^{22} uptake curves. Nerves were soaked in radioactive Ringer's solution at 20°C or at 2°C for periods of time that ranged from 10 minutes to 5 hours. Then the nerves were washed for 1 hour at 2°C in solution W and were analyzed for K, Na, and Na^{22}. The results are presented in Fig. 2, where it is clear that the Na^{22} uptake curves contain at least two components, since the lines do not extrapolate to 100 per cent at zero time. The second component of these curves, which contains about 75 per cent of the Na in the nerves (30 μmols/gm), probably represents intracellular Na, since, at 20°C, it exchanges with a rate constant of about 60 per cent/hr., which is approximately equal to the Na^{22} efflux rate coefficient.[2]

The size of the initial component of these curves is not very sensitive to temperature, since the curves extrapolate to the same point approximately. This component probably represents extracellular Na, and it accounts for about 25 per cent of the Na in these nerves, or 10 μmols/gm. If nerves that have been exposed to Na^{22} for 0.5 hour are washed for 1 hour in cold solution W, they contain 19.3 $\pm$ 2.4 μmols Na^{22}/gm (N = 11). Since the extracellular spaces contain about 10 μmols of this Na^{22}, the intracellular Na^{22} is about 9 μmols/gm. If the nerves are washed in solution W for only 0.5 hour, their Na^{22} contents are 30.1 $\pm$ 5.0 μmols/(N = 68), or 11 μmols/gm higher than

periments were carried out at 2°C, and the loading was carried out at 20°C. The time constant for Na^{22} exchange in the extracellular spaces of toad nerves is about twice as large at 2°C as at 20°C (19). Therefore, at 20°C the intermediate compartment in frog nerve should exchange with a half-time of about 0.25 hour and be 75 per cent exchanged in 0.5 hour. It is doubtful that a change of 25 per cent in the size of the intermediate compartment would be revealed in Fig. 1B.

[2] Results presented in the following paper (7) indicate that about 5 μmols Na/gm remain in the nerve trunk after many hours of soaking in an Na-free solution. Some of this may be due to contamination of the nerve extracts, or it may indicate that a small fraction of the Na is not exchangeable. The data of Fig. 2 are not accurate enough to enable one to decide whether all of the Na of a nerve in Ringer's solution is exchangeable.

in nerves washed for 1 hour. If this additional Na^{22} is assigned to the extracellular compartment, then it appears that, at 2°C, this compartment exchanges with a half-time of approximately 0.5 hour, or a time constant of 0.7 hour, and contains approximately 40 μmols Na^{22}/gm. Clearly, the initial component in Fig. 2 corresponds to the intermediate component in Fig. 1B.

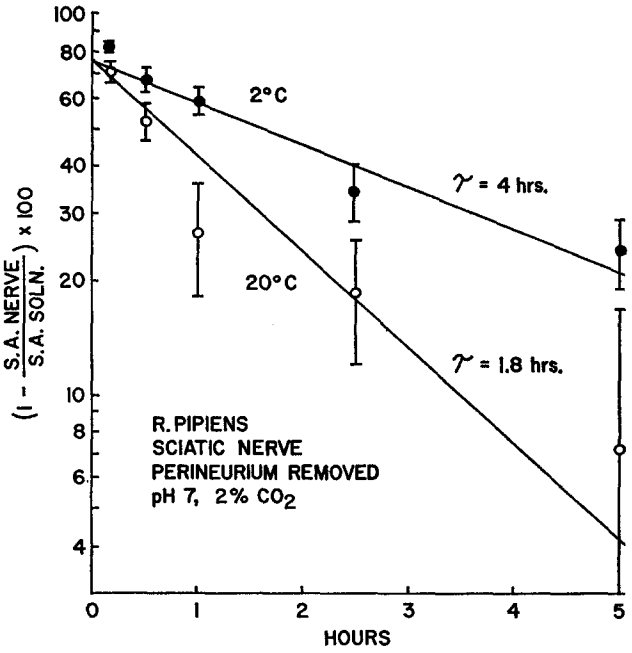

FIGURE 2. Uptake of Na^{22} as a function of time spent in Ringer's solution containing Na^{22}. The quantity

$$100 \times \left[1 - \frac{\text{Specific activity of Na in the nerve}}{\text{Specific activity of Na in the solution}}\right],$$

is plotted on the ordinate using a logarithmic scale. Abscissa, hours in radioactive solution. Each point is an average of five to nine determinations. Vertical bars indicate ± 1 SD. Ruled lines are a visual fit to the data. All nerves were washed for 1 hour at 2°C in solution W before they were extracted for analysis. Mean Na content was 42 ± 7 μmols/gm (N = 66).

These results indicate that after a nerve has been washed for 1 hour at 2°C in solution W, the extracellular Na is about 10 μmols/gm, and that after a nerve has been washed for 0.5 hour, the extracellular Na is about 20 μmols/gm. It is useful to estimate the intracellular Na content and the Na influx and efflux using these data. The Na content of a frog nerve that has been washed for 1 hour in cold solution W is about 40 μmols/gm. Approximately 30 μmols/gm of this total is intracellular Na. The curves of Fig. 1A indicate

that about 20 per cent of the intracellular Na was lost during the hour washing period. Hence, the initial intracellular Na was about 37 μmols/gm. Because of the compensating errors, the result obtained for a 1 hour wash gives a fair estimate of this initial value. The Na^{22} efflux rate coefficient is about 60 per cent/hr., and the efflux, therefore, is 22 μmols/(gm × hr.). When a nerve has been soaked for 0.5 hour in a solution that contains Na^{22},

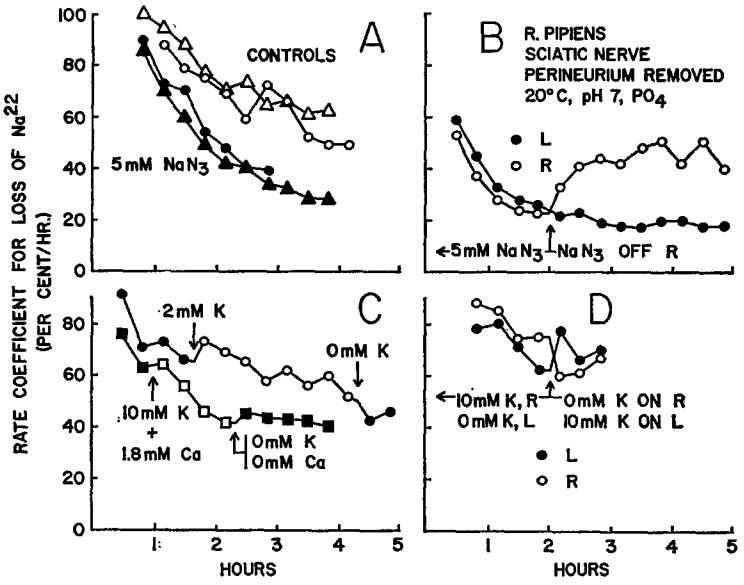

FIGURE 3. Time course of the changes in the Na^{22} efflux rate coefficient obtained from nerves loaded in various ways and washed in various solutions. Ordinate, rate coefficient. Abscissa, time after beginning washout of Na^{22}. Further explanation in text. (A) Effect of NaN_3 on the rate coefficient. (B) Recovery of the rate coefficient from NaN_3 inhibition. Initial washout solution contained 5 mM NaN_3 which was removed from one nerve (R) at the arrow. (C) Effect of K and Ca on the rate coefficient. Upper curve, nerve loaded by soaking 5 hours in a K-free radioactive solution. Lower curve, nerve loaded by soaking 5 hours in a K- and Ca-free radioactive solution. (D) Effect of K on the rate coefficients of paired nerves loaded during stimulation.

and then has been washed for 1 hour in solution W, the intracellular Na^{22} content is about 9 μmols/gm. When this figure is corrected for the 20 per cent loss of intracellular Na^{22} that occurred during the wash period, the estimated Na influx is 22 μmols/(gm × hr.), which agrees with the estimate for the efflux.

Na Efflux Fig. 3 presents some typical results obtained in the efflux studies. Approximately 2 hours were required to clear away the extracellular Na^{22}, and thereafter the efflux rate coefficient assumed a relatively constant value of 40 to 70 per cent/hr. In Fig. 3A, paired nerves were loaded with

Na^{22} by soaking them for 4 hours in radioactive Ringer's solution. One member of the pair was then passed through inactive Ringer's solution, while its partner was passed through a solution that contained 5 mM NaN_3. After a few hours, the NaN_3 reduced the rate coefficient by about 50 per cent. To determine whether the effect of NaN_3 was reversible, paired nerves were loaded with radioisotope by soaking them for 4 to 5 hours in a solution that contained Na^{22} and 5 mM NaN_3. The nerves were then washed in an inactive solution that contained 5 mM NaN_3 (Fig. 3B). When the inhibitor was removed from one of the nerves, the rate coefficient recovered rapidly to a level characteristic of uninhibited tissue. These results are quite conventional and indicate that about half of the Na efflux may be coupled to the cellular metabolism. However, changes in the K concentration of the bathing solution produced only slight effects on the Na^{22} efflux rate coefficient. This is illustrated in Fig. 3C. In these experiments, nerves were soaked for 5 hours in a solution that contained Na^{22} and that lacked either K only or both K and Ca. The efflux was begun in a solution of the same ionic composition as that of the loading solution, and at the first arrow K, or K and Ca, were restored to a nerve and were removed again at the second arrow. These changes in the bathing solution had little effect on the Na^{22} efflux rate coefficient.

The studies of Na efflux from cephalopod giant fibers were carried out with preparations that had been loaded with radioisotope by stimulating them in radioactive solutions (5). In Fig. 3D, paired sciatic nerves were loaded by stimulating the A fibers for 1 hour at 50 volleys per second while the tissue was bathed in radioactive Ringer's solution. Then, one nerve was washed in a solution that contained 10 mM K (five times the normal concentration), and the other nerve was washed in a K-free solution; after 2 hours, the solutions bathing the nerves were interchanged. The efflux rate coefficient obtained with this loading technique, which should be somewhat selective for the large, myelinated fibers, was comparable to that obtained with other methods of loading, and changes in the external K concentration had little effect on the magnitude of this rate coefficient.

Shanes has obtained similar results with toad nerves (16), but he reported a K requirement for nerves that were recovering from anoxia. Under such conditions, the active transport processes may be operating at rates higher than normal, and it might be easier to reveal a K requirement for the Na^{22} efflux. To test this possibility, paired nerves were loaded with Na^{22} in the presence of 5 mM NaN_3. Each of the nerves was then washed in an inactive solution that contained 5 mM NaN_3, but the washout solution used on one nerve contained K at a concentration different from that used on the other nerve. When the NaN_3 was removed after 100 minutes, the degree of recovery of the efflux rate coefficient was independent of the K concentration of the

bathing solution for concentrations up to 60 mM (Fig. 4). The extracellular space should have been well equilibrated with the surrounding solution, since nearly 2 hours had elapsed before the NaN_3 was removed. The fact that 60 mM K had little effect on the Na^{22} efflux rate coefficient indicates that the Na efflux is not influenced by the membrane potential.

The frog sciatic nerve is not a favorable preparation for determining the effects of various agents on the Na^{22} efflux. During the initial 2 hours of an

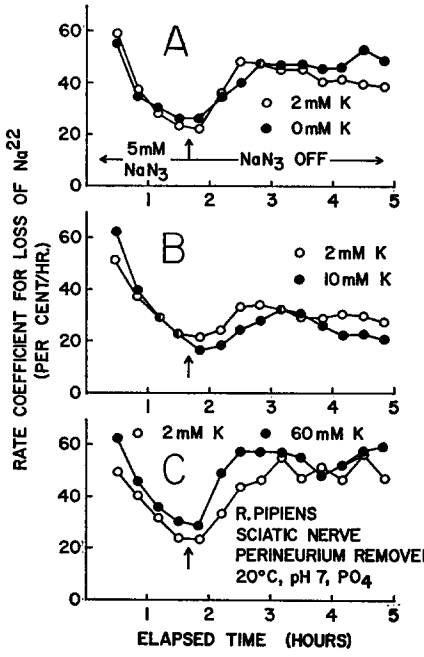

FIGURE 4. Effect of K on the recovery from NaN_3 inhibition of the rate coefficient for Na^{22} efflux. Ordinate and abscissa as in Fig. 3. Initial washout solutions contained 5 mM NaN_3 but different concentrations of K as indicated in the figure. The NaN_3 was removed at the vertical arrow. Further explanation in text.

efflux experiment, most of the Na^{22} comes from the extracellular spaces of a nerve trunk, and the apparent rate coefficient is continually falling; at later times, the quantity of Na^{22} that leaves the nerve is very small. The data in Fig. 4 show that at 20°C the recovery from NaN_3 inhibition is variable and must be carefully controlled. While investigating the effect of temperature on the recovery of the efflux rate coefficient from NaN_3 inhibition, a simple procedure was found that gave a large and quite reproducible change in the efflux rate coefficient. This is illustrated in Fig. 5A. Paired nerves were soaked for 4 hours in a radioactive solution that contained 5 mM NaN_3, and then the nerves were washed at 2°C in an inactive solution that also contained 5 mM NaN_3. When the NaN_3 was removed from the solution that bathed one of the nerves, the efflux rate coefficient did not recover. At the time indicated by the second arrow, both nerves were brought to 20°C. The temperature change had little effect on the efflux rate coefficient of the nerve that was

still in NaN_3, but the rate coefficient of the other nerve increased rapidly to a value characteristic of uninhibited nerve. This result suggests that low temperature and NaN_3 inhibit the same component of the Na^{22} efflux.

The effectiveness of a given agent as an inhibitor of Na transport may be tested by determining whether it can abolish the increase in the Na^{22} efflux rate coefficient that normally occurs when the temperature is raised. The

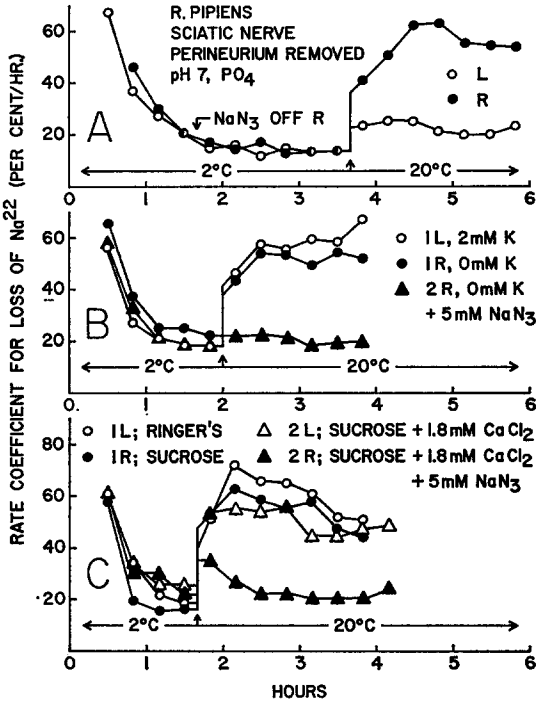

FIGURE 5. Recovery from NaN_3 inhibition of the rate coefficient for Na^{22} efflux. Ordinate and abscissa as in Fig. 3. Nerves loaded by soaking for 4 hours in Ringer's solution containing Na^{22} and 5.0 mM NaN_3. Further explanation in text. (A) Effect of temperature. (B) Effect of K-free solutions. Nerves 1L and 1R were paired. (C) Effect of isotonic (240 mM) sucrose. Paired nerves from two frogs.

effects of a K-free solution are shown in Fig. 5B. These nerves (1L and 1R were from the same frog) were loaded with Na^{22} by soaking them for 4 hours in a radioactive solution that contained 5 mM NaN_3. The washout of the Na^{22} was begun at 2°C. Nerve 1L was in standard Ringer's solution (2 mM K); nerve 1R was in K-free Ringer's solution; and nerve 2R was in a K-free solution that contained 5 mM NaN_3. After 2 hours at 2°C, the nerves were brought to 20°C. Nerves 1L and 1R recovered to the same extent, but the NaN_3 prevented the recovery of nerve 2R. This shows that in a K-free solution the Na^{22} efflux is sensitive to NaN_3.

Fig. 5C shows that the recovery of the efflux rate coefficient can occur when almost all ions have been removed from the bathing solution. These nerves were loaded with Na^{22} in a solution that contained NaN_3, and the washout of the Na^{22} was begun at 2°C. Nerve 1L was in standard Ringer's solution; nerve 1R was in an unbuffered solution of sucrose; nerve 2L was in an unbuffered solution of sucrose plus 1.8 mM $CaCl_2$; and nerve 2R was in unbuffered solution that contained sucrose, 1.8 mM $CaCl_2$, and 5 mM NaN_3. After 100 minutes, the nerves were brought to 20°C. Nerves 1L, 1R, and 2L recovered to normal levels, and the recovery of nerve 2R was prevented by the NaN_3.

A cursory search was made for some of the anions that accompany the Na that is lost from nerves washed in an isotonic solution of sucrose. On six occasions, four nerves were soaked for 2.5 hours at 20°C in an isotonic solution of NaCl, and then were washed for 1 hour at 2°C in a large volume of an isotonic solution of sucrose. The four nerves were then transferred to 2 ml of an isotonic solution of sucrose at 20°C and were left for 2 hours. The second sucrose solution was analyzed for K, Na, Cl, and the sum of creatine phosphate and inorganic phosphate [by a modified method of Fiske and SubbaRow (13)], and the pH was checked with a pH meter. The results (in μmols/gm dry nerve) were K = 10, Na = 67, Cl = 13, and $CrP + P_i = 16$. The pH of fresh samples of the isotonic sucrose solution, to which 0.1 mM NaCl had been added, ranged from 5.5–5.8; the pH of the sucrose solution in which the nerves had been washed, ranged from 6.1–6.5. It is clear that many of the anions that left the nerves were not identified.

Dinitrophenol at a concentration of 0.2 mM is about as effective as 5 mM NaN_3 in preventing the increase in the Na^{22} efflux rate coefficient that occurs when the temperature is raised from 2 to 20°C. This action of dinitrophenol can occur in a K-free solution.

Ouabain is a potent inhibitor of the Na efflux from the squid giant axon (1), but not of the Na efflux from frog nerve. This is shown in Fig. 6. The nerves in Fig. 6A were loaded with Na^{22} by soaking them for 4 hours in a K-free radioactive solution that contained 5 mM NaN_3, and the washout of the Na^{22} was begun at 2°C. Nerve 1L (open circles) was in standard Ringer's solution; nerve 1R (filled circles) was in Ringer's solution that contained 0.05 mM ouabain; nerve 2L (open triangles) was in K-free Ringer's solution; and nerve 2R (filled triangles) was in K-free Ringer's solution that contained 0.05 mM ouabain. After 2 hours, the nerves were brought to 20°C. The ouabain reduced the efflux rate coefficient by about 25 per cent. This inhibition of the Na^{22} efflux was not dependent on the external K concentration. This is shown more clearly in Fig. 6B. These nerves were loaded with Na^{22} under the same conditions used for the nerves in Fig. 6A, and the washout of the Na^{22} was begun at 2°C. Nerves 1L (open circles) and 2R (open triangles) were in

standard Ringer's solution that contained 0.05 mM ouabain, and nerves 1R (filled circles) and 2L (filled triangles) were in a K-free solution that contained 0.05 mM ouabain. After 2 hours, the nerves were brought to 20°C. The curves for the nerves in the K-free solution are virtually indistinguishable from the curves for the nerves in standard Ringer's solution.

Because of the small effect of ouabain, it was difficult to obtain a concentration-effect curve. Increasing the ouabain concentration to 0.5 mM did not markedly increase the inhibition of the Na^{22} efflux. Somewhat erratic

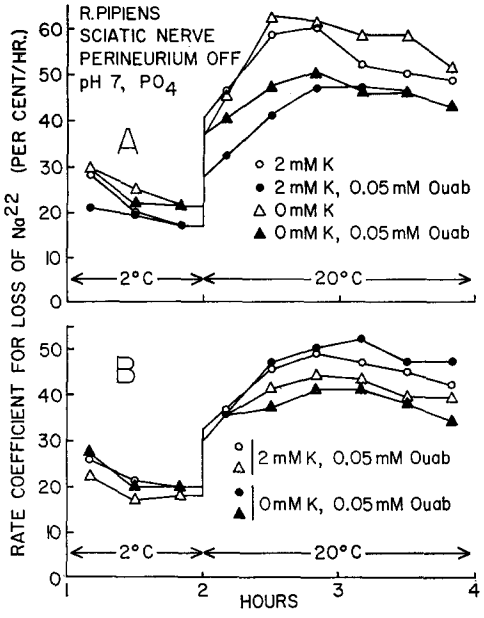

FIGURE 6. Effect of 0.05 mM ouabain on the response of the Na^{22} efflux rate coefficient to changes in temperature. Nerves loaded in 5 mM NaN_3. Ordinates and abscissas as in Fig. 3. Further information in text. (A) Inhibition produced by ouabain in Ringer's solution or in K-free Ringer's solution. (B) Recovery of paired nerves in the presence of 0.05 mM ouabain, one in Ringer's solution and one in K-free Ringer's solution. The filled circles were omitted for times earlier than 2 hours, because they coincided with the filled triangles.

effects on the Na^{22} efflux were obtained with 0.01 mM ouabain. On some occasions, the inhibition was similar to that shown in Fig. 6A; on other occasions, there was almost no effect. Ouabain at a concentration of 0.01 mM strongly inhibited K^{42} uptake by a nerve (7) and produced significant changes in the Na and K contents of a nerve at a concentration of 0.001 mM (see below).

The above results indicate that the efflux of Na from frog nerve is only slightly inhibited by a K-free solution. Since such a solution causes a nerve to gain Na, it must increase the Na influx into the nerve.

Na Influx Nerves were soaked for periods up to 5 hours in solutions that contained 5 mM NaN_3, in K-free solutions, or in K- and Ca-free solutions. After having been soaked in one or another of these solutions, some nerves were allowed to recover in a solution that contained the normal complement of Ca and that contained K at a concentration of 0, 2, or 10 mM. The Na

and K contents of the nerves and the initial rates of Na^{22} uptake were determined at various times during the course of these experiments. The results are summarized in Figs. 7 to 9.

The following procedure was used to follow the changes in the initial rates of Na^{22} uptake that were produced by the various solutions. To determine the immediate effects of a given experimental solution, a nerve was soaked for about 2 hours in Ringer's solution, and then it was soaked for 0.5 hour in an aliquot of the experimental solution that contained Na^{22}. Next, the nerve was washed for 0.5 hour at 2°C in solution W, and then it was dried, weighed, and extracted. To determine the initial rate of Na^{22} uptake after 2.5 hours in a given experimental solution, a nerve was soaked for 2 hours in that experimental solution, and then it was soaked for 0.5 hour in an aliquot of the experimental solution that contained Na^{22}. Then the nerve was washed in solution W, dried, weighed, and extracted. To determine the rate of Na^{22} uptake after 5 hours in a given experimental solution, a nerve was soaked in that solution for 4.5 hours before it was transferred for 0.5 hour to an aliquot of the experimental solution that contained Na^{22}. To measure the initial rate of Na^{22} uptake during the recovery period, a nerve was soaked in the experimental solution for 2.5 hours, or for 5 hours, before it was transferred to the recovery solution for the appropriate length of time.

Most of the nerves were analyzed for Na, K, and Na^{22}, but, because Na and K appeared to exchange on an equimolar basis, only the Na data are presented here. Usually, measurements were made with paired nerves, with one nerve serving as a control, but only the averages of the values of the Na and Na^{22} contents of the nerves are shown; similar curves were obtained if differences were plotted. Remember that the extracellular spaces contained about 20 μmols/gm of the Na and Na^{22} in these nerves. For the control nerves, the average values (in μmols/gm) of the ionic contents and of the amount of Na^{22} taken up in 0.5 hour were: in standard Ringer's solution: K = 160 $\pm$ 11, Na = 48 $\pm$ 6 (N = 56), and Na^{22} = 30.1 $\pm$ 5.0 (N = 68); in Ringer's fluid that contained K at a concentration of 10 mM: K = 168 $\pm$ 7, Na = 49 $\pm$ 4, and Na^{22} = 28.2 $\pm$ 2.2 (N = 12). These average values are indicated by the dashed lines in each of the figures (Figs. 7 to 9). The points plotted at 5 hours in Figs. 7, 8, and 9 and the point plotted at 2.5 hours in Fig. 8A are averages of twenty-nine or more measurements. The other points are averages of from three to six analyses.

Fig. 7 illustrates the effects produced by a K-free solution. The upper portion of Fig. 7 shows the progressive increases in the Na contents that occurred while the nerves were soaking in such a solution. If after 5 hours in a K-free solution, some nerves were placed in Ringer's solution (2 mM K) their Na contents fell to normal in about 5 hours. If the recovery solution contained 10 mM K, the Na contents recovered somewhat more rapidly.

The lower part of Fig. 7 illustrates the changes in the rates of Na^{22} uptake that occurred during these experiments. During the first half hour that the nerves were in a K-free solution their Na influxes were increased by 24 ± 12 μmols/(gm × hr.) (N = 6). The increases in the Na influxes grew larger the longer the exposure to the K-free solution. If some nerves were returned to Ringer's solution after 5 hours, their influxes fell abruptly by about 22 μmols/(gm × hr.) to values between the values in the K-free solution

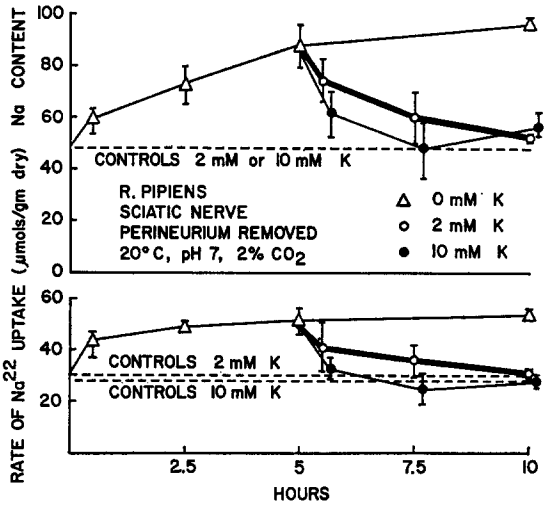

FIGURE 7. Changes in the Na content and the initial rate of Na^{22} uptake that occur during soaking in K-free solutions and during recovery therefrom. Ordinate, upper curve, Na content; lower curve, Na^{22} taken up in 0.5 hour. Abscissa, time since beginning the exposure to K-free solutions. In this figure and in Figs. 8 and 9, the symbols indicate the K concentrations of the bathing solutions. Vertical bars indicate ± 1 SD, or the range of the values when the mean was based on three analyses. During the recovery phase, the points were slightly displaced along the abscissa to avoid overlap of the bars indicating the SD. All nerves were washed 0.5 hour at 2°C in solution W before they were extracted for analysis. The extracellular spaces contain about 20 μmols/gm of the Na and Na^{22} in the nerves.

and the values in standard Ringer's solution, and then gradually returned to the control level. The rapid initial changes in the Na influxes are about equal to the normal K influx of 20 μmols/(gm × hr.) (7). When the recovery solution contained 10 mM K, the initial decreases in the rates of Na^{22} uptake were greater.

In Fig. 8A the nerves were first soaked in a K- and Ca-free solution, and after 2.5 hours were allowed to recover in a solution that contained Ca at the standard concentration. The K- and Ca-free solution produced very large increases in the Na contents of the nerves and in their rates of Na^{22}

uptake. It is not known to what extent spontaneous electrical activity may have contributed to these changes. When the nerves recovered in a K-free solution, their rates of Na^{22} uptake fell to values considerably greater than normal and remained there. The Na contents did not recover, but the rates of gain of Na were slowed. When nerves recovered in standard Ringer's solution, their Na^{22} influxes fell abruptly to values much greater than normal and eventually declined to the control values. Their Na contents recovered

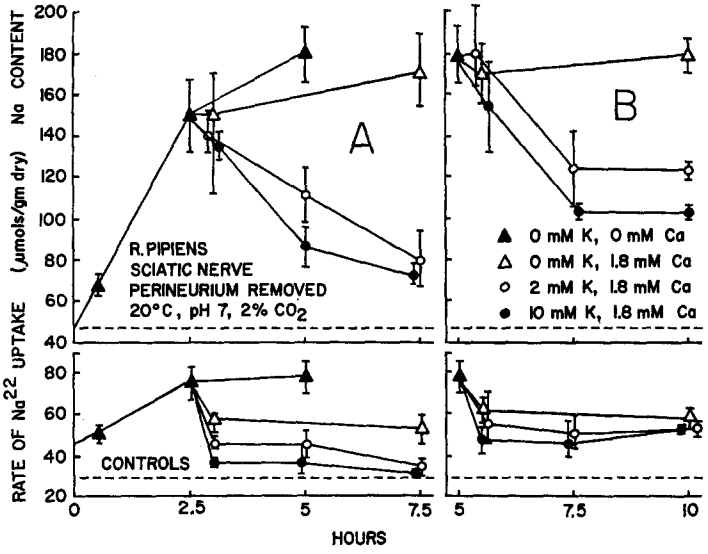

FIGURE 8. Changes in the Na content and the initial rate of Na^{22} uptake that occur during soaking in K- and Ca-free solutions and during recovery therefrom. Ordinates and abscissas as in Fig. 7. In A recovery started after 2.5 hours and in B after 5 hours.

readily. When the recovery solution contained 10 mM K, the Na contents of the nerves recovered slightly more rapidly, and the initial decreases in the rates of Na^{22} uptake were greater than was the case with standard Ringer's solution.

Fig. 8B illustrates the recovery that occurred after nerves had been soaked for 5 hours in a K- and Ca-free solution. The recovery of the Na contents appeared to be incomplete, and the rates of Na^{22} uptake remained greater than normal in each of the recovery solutions.

The effects of 5 mM NaN_3 are illustrated in Fig. 9. This substance produced increases in the Na contents of nerves; these changes were slowly reversed when the nerves recovered in standard Ringer's solution. The recovery of the Na contents of the nerves was somewhat faster when the recovery solution contained 10 mM K, and the recovery of the ionic contents was prevented by a K-free solution. When nerves were soaked in standard Ringer's solution

that contained 5 mM NaN_3, their rates of Na^{22} uptake were increased slightly. When such nerves recovered in standard Ringer's solution, their rates of Na^{22} uptake first underwent additional small increases and then slowly returned to normal. If the recovery solution contained no K, the rates of Na^{22} uptake increased considerably and remained elevated.

Dinitrophenol (0.2 mM) or 0.001 mM ouabain also produced changes in the ionic contents of nerves and produced increases in their rates of Na^{22}

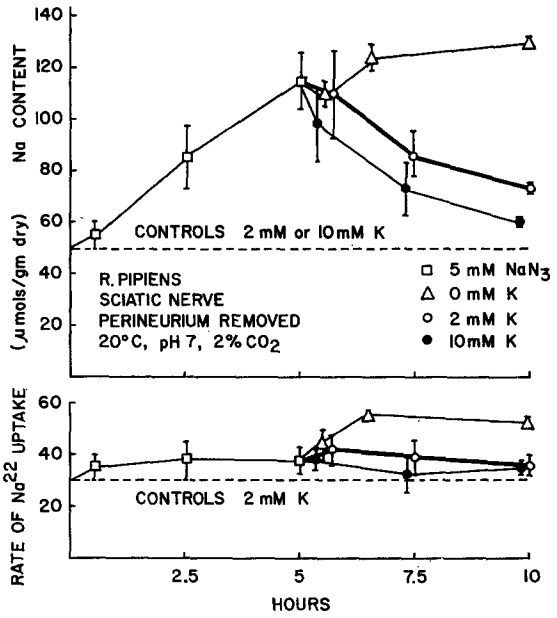

FIGURE 9. Changes in the Na content and the initial rate of Na^{22} uptake that occur during soaking in Ringer's solution containing 5 mM NaN_3 and during recovery therefrom. Ordinate and abscissa as in Fig. 7.

uptake. Table I shows the changes in the ionic contents and in the rates of Na^{22} uptake that occurred after nerves had been soaked for 4.5 to 5 hours in a solution of one of these inhibitors. During the first half hour that nerves were exposed to 0.2 mM dinitrophenol or to 0.001 mM ouabain the amount of Na^{22} taken up in 0.5 hour was increased by 4.9 ± 0.3 μmols/gm (N = 3), or by 4.7 ± 0.9 μmols/gm (N = 3), respectively. The effects of 0.2 mM dinitrophenol on the rates of Na^{22} uptake and on the ionic contents were similar to the effects of 5 mM NaN_3. Dinitrophenol had little effect when used at a concentration of 0.02 mM. Ouabain also increased the rates of Na^{22} uptake about as much as did 5 mM NaN_3, but ouabain produced changes in the ionic distributions that were much smaller than those produced by NaN_3. This is because ouabain has only a slight effect on the Na efflux and 5 mM NaN_3 reduces the Na efflux by about 50 per cent.

If the figure in Table I for the increase in the amount of Na^{22} taken up in 0.5 hour is multiplied by 10 to estimate the gain in Na that should have occurred in 5 hours, the result for a nerve soaking in 0.001 mM ouabain is about 66 μmols/gm, or about twice as large as the observed change in the Na content. This is not an inconsistency in the data, for this calculation assumes that the Na efflux and influx remained constant over the experimental period. However, since ouabain has little effect on the Na efflux rate coefficient, the efflux must have increased as the Na content rose. The amount of Na^{22} taken up in 0.5 hour by normal nerve is about 10 μmols/gm. Ouabain increases this about 66 per cent. If the Na content of a nerve increased by 66 per cent (about 26 μmols/gm), the nerve would be in a new steady state in the ouabain solu-

TABLE I

EFFECT OF OUABAIN OR DINITROPHENOL ON THE IONIC CONTENTS AND THE Na INFLUXES IN NERVES IN RINGER'S SOLUTION

Inhibitor	Concentration	Change in		
		K content	Na content	Na^{22} uptake—0.5 hr.*
	mM			
Ouabain	0.001	−26±5 (9)	+29±5 (9)	+6.6±2.6 (5)
Ouabain	0.01	−31±10 (10)	+33±6 (10)	+6.4±2.2 (4)
α-DNP	0.2	−64±10 (10)	+55±7 (10)	+7.7±3.2 (6)

Mean values ± SD. Units are μmols/gm.

The figures in parentheses indicate the number of pairs of nerves that contributed to each average value.

* The nerves were soaked for 4.5 to 5 hours in their respective solutions, and the rate of Na^{22} uptake was measured over the last half-hour interval. The figures listed in this column are the differences between the Na^{22} contents of the experimental and the control nerves. These numbers should be doubled to get the change in the Na influx.

tion, at least as far as its Na content was concerned. This calculated increase in the Na content corresponds well with the observed increase in the Na content, and this result indicates that the observed changes in Na influx account for the observed increases in the Na content, if it is assumed that ouabain has little effect on the Na efflux.

The general impressions given by these results are that, except for those solutions that contained metabolic inhibitors, the various experimental solutions had little effect on the Na efflux from frog nerve, and that the primary cause of the changes of the Na contents was the changes in the Na influxes. It is important to determine whether the measurements of the Na influxes and the Na effluxes form a self-consistent set of observations and account for the net movements of Na in the nerves.

The efflux studies indicate that K-free, or K- and Ca-free solutions have no pronounced effect on the Na^{22} efflux rate coefficient of a nerve, and that

5 mM NaN_3 produces an effect only after several hours. Yet, during the first half hour that nerves are soaked in one of these solutions, their Na contents are increased. If the initial effect of these solutions is to increase the Na influx only, then during the first half hour of soaking, one should observe that the change in the Na content of a nerve is equal to the change in the Na^{22} uptake. The data of Fig. 10 show that this is the case. Paired nerves were soaked for several hours in standard Ringer's solution. One nerve was then transferred for 0.5 hour to Ringer's solution that contained Na^{22} while its mate was placed for 0.5 hour in a modified Ringer's solution that also contained Na^{22}. Then both nerves were washed for 0.5 hour at 2°C in solution

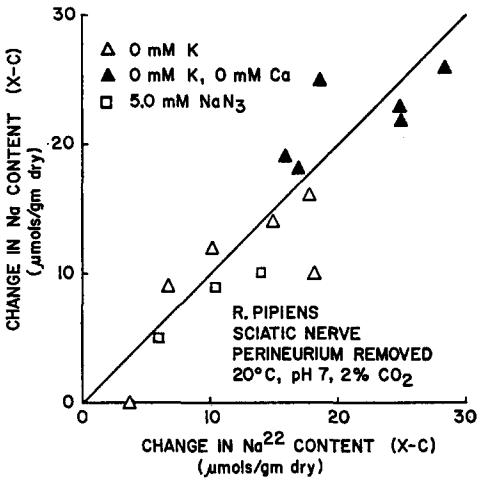

FIGURE 10. Changes in the Na content and the Na^{22} uptake that occur during the first half hour of exposure to various solutions. Paired nerves. Ordinate, difference between the Na content of an experimental nerve soaked in modified Ringer's fluid and that of a control nerve soaked in standard Ringer's fluid. Abscissa, difference between the Na^{22} contents of the same pair of nerves. Symbols indicate modifications of the various solutions.

W and prepared for analysis. The difference between the Na contents of the members of each pair of nerves is plotted on the ordinate of Fig. 10, and the difference between their Na^{22} contents is plotted on the abscissa. The increase in the Na content is equal to the increase in the Na^{22} content.

A more extensive, but less sensitive, check of the data is indicated below. If the axoplasm is the only compartment in a nerve, then according to the definitions of the Na influx and efflux, the rate of change of Na in the axoplasm is given by the equation:

$$\frac{dQ_i}{dt} = M_i - M_o$$

where the symbols have the meanings assigned earlier.

From equation (3):

$$M_o = kQ_i$$

where k is the Na^{22} efflux rate coefficient for isotope movement from the axoplasm.

Solving these equations for k:

$$k = \frac{M_i - dQ_i/dt}{Q_i}$$

The entities on the right side of this equation are available from Figs. 7 to 9. If the data are self-consistent, the factor k computed in this way should have a value of approximately 60 per cent/hr., be independent of the Na contents of the nerves, and be independent of the composition of the external solution, except that in 5 mM NaN_3 it should be reduced about 50 per cent.

At any particular time, dQ_i/dt was computed as the average of the slopes of the two intersecting straight lines that are drawn through the experimental points in Figs. 7 to 9. After having been washed for 0.5 hour at 2°C in solution W, a nerve contains approximately 20 μmols Na^{22}/gm in an extracellular compartment. Therefore, the data of Figs. 7 to 9 overestimate the intracellular Na and Na^{22} by this amount, and the formula used to compute k was:[3]

$$k = \frac{[(Na^{22}\text{ uptake} - 20) \times 2 - \overline{\Delta Q_i/\Delta t}]}{Q_i - 20}$$

The results of these calculations are summarized in Fig. 11. The mean value of k, excluding the points in 5 mM NaN_3, was 69 ± 17 per cent/hr. The computed values of k behave as expected, although there may be some tendency for the value of k to fall off at very high internal Na levels. The data at high levels of internal Na were obtained from nerves that had been soaked for more than 5 hours in K- and Ca-free solutions. A low value of k is implied also by the data in Fig. 8B, where it is clear that the difference among the Na contents of the nerves cannot be accounted for entirely by the differences among the rates of Na^{22} uptake. These are severe treatments, and the results do not undermine the primary point of this report: that the Na efflux is not strongly dependent on the external K concentration.

The correspondence between the rate coefficient observed in the efflux experiments and the rate coefficient calculated from the influxes and the changes in the Na content is important for the following reason. It might be supposed that the major fraction of the Na efflux represented a self-exchange of Na that did not contribute to the net Na movements, and that this self-exchange component of the efflux obscured the presence of a small active

[3] The curves of Fig. 1 indicate that about 10 per cent of the intracellular Na and Na^{22} were lost during the washing period, and the Na and Na^{22} contents should be corrected for this. Since the same correction factor would be applied to each of the terms in the above formula, it would cancel out.

component that was coupled to K transport. However, the results obtained for the computed value of the efflux rate coefficient show that, at least to a first approximation, the net movements of Na can be accounted for by the measured changes in the Na influx, if it is assumed that, in the absence of metabolic inhibition, the Na efflux is directly proportional to the Na content and is independent of the K concentration of the bathing solution. If there be a small component of the Na efflux that is linked to the K influx, it does not make a significant contribution to the observed changes in the Na content.

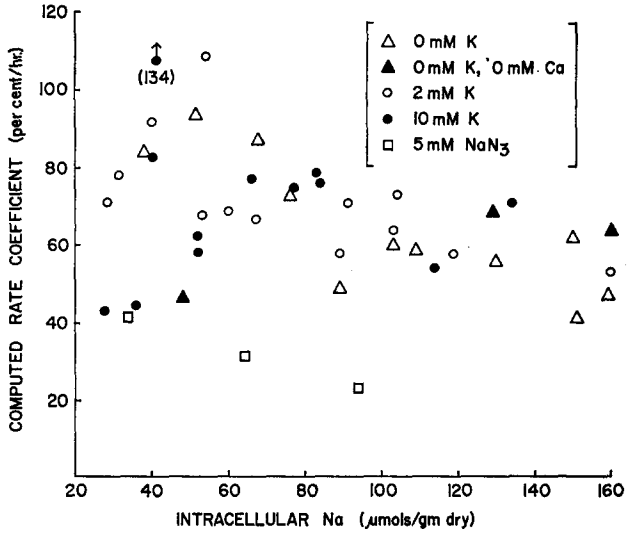

FIGURE 11. Values of k, the rate coefficient for Na^{22} efflux, computed from the data of Figs. 7 to 9 according to the formula given in the text. Ordinate, computed values of the rate coefficient. Abscissa, mean values of the intracellular Na content of the nerves for which the various values of k were computed. The intracellular Na content is equal to the measured Na content minus 20 (see text).

It is useful to examine in more detail the changes in the Na influx and the Na efflux that occur during the procedures used here. When a nerve is in Ringer's solution, the Na influx and efflux are 20 μmols/(gm × hr.), approximately. The Na^{22} efflux rate coefficient is 40 to 70 per cent/hr., and in the following calculations a value of 60 per cent/hr. will be used.

The initial effect of a K-free solution is to increase the Na influx into a nerve, to leave the Na efflux unchanged, and thus to cause the nerve to gain Na. As the Na content rises, the Na efflux rises proportionally. After 5 hours in a K-free solution, the Na influx is approximately 62 μmols/(gm × hr.) [(51 − 20) × 2], and the Na efflux is about 40 μmols/(gm × hr.) [(87 − 20) × 0.6]. Both the influx and the efflux of Na are two to three times higher than normal, but since the Na influx exceeds the Na efflux, the nerve continues to

gain Na in the absence of K. If the nerve is transferred to Ringer's solution, the Na influx drops sharply to 40 μmols/(gm $\times$ hr.)[(40 $-$ 20) $\times$ 2], a value somewhat higher than normal and equal to the elevated Na efflux. A slight increase in the efflux rate coefficient is required for the nerve to lose Na. The curves in Figs. 3 and 4 indicate that there may be a small effect of external K on the Na^{22} efflux rate coefficient. However, the major effect of external K is on the Na influx.

The effects of K and Ca lack (Fig. 8) are an exaggerated version of simple K lack. After a nerve has been soaked for 2.5 hours in isotonic NaCl, the Na influx is 110 μmols/(gm $\times$ hr.)[(75 $-$ 20) $\times$ 2], and the Na efflux is 77 μmols/(gm $\times$ hr.)[(149 $-$ 20) $\times$ 0.6]. If Ca is restored to the external solution, the Na influx falls to 76 μmols/(gm $\times$ hr.)[(58 $-$ 20) $\times$ 2], a value close to the Na efflux and no recovery of the ionic distribution occurs. When the nerve recovers in normal Ringer's, the Na influx falls to 52 μmols/(gm $\times$ hr.) [(46 $-$ 20) $\times$ 2], which is considerably less than the Na efflux, and Na is lost from the nerve. The failure of nerves to recover completely after they have been soaked for 5 hours in isotonic NaCl is correlated with a permanently elevated Na influx (Fig. 8B).

Azide has two effects. Its immediate effect is to increase the Na influx, and after 5 hours the influx is about 34 μmols/(gm $\times$ hr.)[(37 $-$ 20) $\times$ 2]. The NaN_3 also reduces the Na^{22} efflux rate coefficient to 20 to 30 per cent/hr. in this time, and increases the Na content to about 100 μmols/gm. After 5 hours in 5 mM NaN_3, therefore, the Na efflux is 20 to 30 μmols/(gm $\times$ hr.), which is little different from normal, and still is less than the Na influx. The nerve continues to gain Na if left in the solution of NaN_3. When the NaN_3 is removed, the efflux rate coefficient recovers to a value of 50 to 60 per cent/hr. (Figs. 4 and 5), and the Na efflux at this time should be 50 to 60 μmols/(gm $\times$ hr.), approximately three times normal. During the first half hour of recovery in Ringer's solution, the Na influx rises further to 42 μmols/(gm $\times$ hr.) [(41 $-$ 20) $\times$ 2], a rate approximately twice normal, but less than the elevated Na efflux, and the nerve loses Na. When the recovery occurs in a K-free solution, the efflux rate coefficient attains its normal value and the Na efflux is, therefore, about 60 μmols/(gm $\times$ hr.). Fig. 9 shows that, under these conditions, the Na influx increases to 66 μmols/(gm $\times$ hr.)[(53 $-$ 20) $\times$ 2], a value slightly higher than the Na efflux, and the nerve continues to gain Na. The failure of a nerve to lose Na when recovering from NaN_3 inhibition in a K-free medium is not due to a reduced Na efflux, but is due to an increased Na influx.

The results presented here may be summarized as follows. The Na efflux from frog nerve is directly proportional to the Na content of the nerve and is independent of the K concentration of the bathing solution; about 50 per cent of the Na efflux appears to be dependent on the metabolism, and perhaps

25 per cent of the efflux may be inhibited by ouabain. If there is a component of the Na efflux that is coupled to the K influx, it is small and appears not to play a significant role in determining the ionic distribution in frog nerve. The Na influx is variable and appears to adopt whatever value is necessary to insure that the net movements of Na are equal in magnitude, but opposite in direction, to the net movements of K.

Cl Content A few experiments were done to determine the changes that occurred in the Cl contents of nerves soaked in solutions containing 5 mM NaN_3 or in solutions lacking K and Ca. For each determination, paired nerves from two frogs were used; one nerve from each animal served as a control, while the others were soaked in one of the experimental solutions. The nerves were washed for 0.5 to 1 hour at 2°C in a solution that contained 118 mM $NaNO_3$ and 1.8 mM Ca $(NO_3)_2$ before they were dried and extracted. The time constant for the loss of Cl from nerves in this washing solution was about 5 hours when measured over the time interval from 0.5 to 2 hours. The time constant for Cl^{36} exchange in toad nerves in Ringer's solution at 4°C is about 10 hours (19). These results indicate that the washing procedure reduced the Cl content of the axoplasm by less than 20 per cent. The Cl titrations were not affected by quantities of $NaNO_3$ several times larger than the quantity that was estimated to have been contained in the extracellular spaces of the nerves. When nerves were soaked for 5 hours in a K- and Ca-free solution, their Cl contents increased 12 ± 4 μmols/gm (N = 3). During 5 hours in a solution that contained 5 mM NaN_3, the Cl contents decreased 3 ± 2 μmols/gm (N = 3). These changes are small relative to the changes in the Na and K contents that occur under these conditions. The average value of the Cl contents of the control nerves was 34 ± 6 μmols/gm (N = 6).

DISCUSSION

Several explanations could be invoked to account for the results presented here: (*a*) failure to wash the extracellular spaces free of K; (*b*) uncertainty as to the anatomical location of the Na^{22}; (*c*) the possibility that active Na efflux is reduced by a K-free solution but that the reduction is masked by a concomitant increase in passive Na permeability; (*d*) complications due to the slowness of diffusion within the axoplasm.

The first of these explanations can be discounted for two reasons. In most of the efflux experiments, the movements of Na^{22} were followed for several hours in K-free solutions. This should have been ample time for appreciable changes in the concentration of K in the extracellular space to have occurred. From an analysis of afterpotentials recorded from *isolated* nerve fibers of the frog, Meves (11) has concluded that no structure is present in the nodal region that delays significantly the diffusion of K from the perinodal space to the

bathing solution. More important is the observation that during the first half hour that a nerve is washed in a K-free medium the Na content and the Na influx increase. This indicates that changes do occur in a short time; the problem is to explain why they are revealed as changes in Na influx rather than as changes in Na efflux. It is difficult to see how such an effect could be produced by a failure to clear the extracellular spaces of K.

Without histochemical controls, the second explanation cannot be answered definitely. It can be pointed out, however, that the Na^{22} efflux rate coefficient is not dependent on the method used to load a nerve, except when this coefficient is determined in the presence of NaN_3. With some of the loading procedures 50 to 75 per cent of the K in the nerves was exchanged for Na. It appears that the Na movements being studied are those to and from the axoplasm of the myelinated nerve fibers.

Shanes suggested (16), and later rejected (17), the third explanation to account for the failure of anoxia to inhibit the Na efflux from toad nerve. This explanation cannot be excluded entirely in the present instance. However, there is no indication that changes in passive Na permeability produce changes in the Na^{22} efflux rate coefficient. Ca-free solutions are believed to increase the permeability of nerve fibers to Na (14), and in the absence of K and Ca, the Na influx increases fivefold (Fig. 8). However, the Na^{22} efflux rate coefficient is not changed markedly by such solutions. Therefore, it appears that changes in passive Na permeability are not reflected in the Na^{22} efflux rate coefficient, and it seems unlikely that the lack of effect of K-free solutions on the Na^{22} efflux rate coefficient is the result of opposing actions on active transport and on passive permeability.

The final objection is also difficult to dismiss completely. Conventional isotope exchange theory assumes that there is good mixing on both sides of the membrane. While this may be valid for most cells, the nodal structure of heavily myelinated vertebrate axons poses special problems. In these axons, it is possible that isotope exchange occurs largely at the nodes. After having crossed the nodal membrane, radioactive material would have to distribute itself along the relatively long internode, and appreciable concentration gradients of radioactive material might build up within the axoplasm and render inapplicable the conventional interpretation of radioisotope data.

Consider an infinite plane sheet of thickness $2l$ containing a substance, S, that is distributed uniformly within. If this sheet is placed suddenly in a large volume of solution that is well stirred and that contains no S, then diffusion theory predicts that the amount of S remaining within the sheet would decline with a final time constant, $\tau = 4l^2/\pi^2 D$ (2, page 45), where D is the diffusion coefficient of S.

This system is analogous to the diffusion of Na^{22} from an internode of length $2l$ through the two adjacent nodes, if the myelin sheath is assumed

to be impermeable. In this case, $l = 1$ mm and D is the diffusion coefficient for Na in the axoplasm. If the mobility of Na in the axoplasm were the same as its mobility in aqueous solution ($D = 1.3 \times 10^{-5}$ cm²/sec.), then in the absence of any impediment to diffusion at the nodes, the relaxation of the Na^{22} content should occur with a final time constant of 5.2 minutes. The observed time constant is about 100 minutes, nearly 20 times longer. To explain completely the time course for Na^{22} exchange on the basis of internodal diffusion requires that the mobility of Na in the axis cylinder be about 5 per cent of the mobility of Na in aqueous solution.

A closer analogue of diffusion in a single fiber is that of diffusion in an infinite plane sheet where material is transferred across the surface of the sheet at a rate proportional to the surface concentration. If such a model is assumed to represent diffusion in a single fiber, then it can be shown that the mobility of Na within the axoplasm must be less than 18 per cent of the mobility of Na in aqueous solution, if internodal diffusion is to interfere seriously with measurements of Na fluxes across the nodes (see Appendix).

Müller (12) recorded potential changes that occurred during the electrical polarization of single fibers that were rich in Na and deficient in K. He interpreted the results to mean that the mobility of Na in the axoplasm is only 5 to 10 per cent of the mobility of Na in aqueous solution. It is desirable to obtain a reliable estimate of the diffusion coefficient of Na in the axoplasm of frog fibers. It seems unlikely that the mobility of all intracellular monovalent cations is low, for the efflux of K^{42} from frog nerves is increased about sixfold when the external K concentration is increased twenty times (17). This indicates that the efflux of K is strongly influenced by the membrane potential and suggests that for K ions, at least, diffusion within the axoplasm is not the rate-determining step in ionic movements.

It cannot be decided definitely how the various solutions produce an increase in the Na influx. It seems unlikely that changes in the membrane potential are responsible, for to increase the influx of a cation, the membrane potential must increase in magnitude. K-free solutions increase the membrane potential only a few millivolts (9) and Ca-free solutions or solutions that contain metabolic inhibitors produce depolarization (14). When Ca-free solutions are used, spontaneous electrical activity may produce some of the increase in the Na influx.

The increases in the Na influx could be ascribed solely to increases in the Na permeability of the membrane, and this may be the correct interpretation of the effects of a Ca-free solution, but the fact that the net uptake of Na by a nerve is almost always balanced by an equivalent loss of K from the nerve indicates that the changes in the influx and the efflux of Na and the changes in the influx and the efflux of K do not occur independently of one another,

but are subject to some kind of constraint, perhaps the need to maintain electroneutrality.

When a nerve is first placed into a K-free solution and again when it is first transferred back to Ringer's solution, the changes in the Na influx are approximately equal to the K influx in resting nerve. This certainly suggests that some of the changes in the Na influx are related directly to changes in the K influx. Not all the changes in the Na influx can be explained in this way, however. When nerves are recovering in Ringer's solution after having been soaked for several hours in a solution that contained 5 mM NaN_3, or in a K-free solution, or in a K- and Ca-free solution, the Na influx and the Na efflux are both several times greater than normal, the K efflux is less than normal (7), and the K influx is normal or slightly greater (7). Under such conditions, the high Na influx may be due to an increase in the Na permeability of the membrane, or it may represent a kind of self-exchange in the sense that the net Na movements are small although the Na efflux and influx are large. This self-exchange of Na is not obligatory, since the Na efflux proceeds quite well in isotonic sucrose (Fig. 5).

Shanes's suggestion of an Na exclusion process (17) is one possible explanation for some of the increases in the Na influx. According to this idea, most of the Na ions that enter the membrane from the external solution are extruded in exchange for extracellular K ions before they enter the main body of the axoplasm. Since the extruded Na ions have not come from the axoplasm, inhibition of the exclusion process leads to an increase in the Na influx rather than to a decrease in the Na efflux. If the exclusion process is located at the inner side of the cell membrane, it is difficult to see how this system differs from the conventional Na-K exchange pump. If the exclusion process is located on the external surface of the cell membrane, it is not meaningful to speak of an influx of Na or of an exchange between extracellular Na and extracellular K. If the exclusion process is located within the membrane, then it must be assumed that the rate-limiting step in the active transport of K and Na ions occurs between the axoplasm and the place where the extrusion process is located. This would mean that the rate of turnover of the exclusion pump was much greater than is indicated by the flux measurements in this paper, and such a process would be an inefficient way of maintaining the ionic distribution. Nevertheless, the presence of an Na exclusion pump is one explanation of the present results. It should be noted that if the mobility of Na in the axoplasm were very low, then diffusion within the axoplasm itself would constitute the rate-limiting step for ion movement into or out of the fibers and would produce the situation whereby an inhibition of the ion pump would lead to an increase in the Na influx.

According to Shanes's hypothesis, any inhibition of the exclusion pump will produce an increase in the Na influx. If it is supposed that NaN_3 inhibits

completely the Na exclusion pump, then the increase in the Na influx produced by NaN_3 should be as great as that produced by a K-free solution. Figs. 7 and 9 show that the increase in the Na influx that is produced by 5 mM NaN_3 (in Ringer's solution) is less than the increase that is produced by a K-free solution. This might indicate that NaN_3 reduces the passive Na permeability of the barrier that is presumed to lie between the exclusion pump and the axoplasm, and the decrease in the passive permeability of this structure might be the cause of the reduction in the Na efflux that is produced by NaN_3. (Note that this requires that the passive permeability of the structures that lie between the exclusion pump and the extracellular solution be much greater than the passive permeability of the structures that are located interior to the exclusion pump.) To check this possibility, four pairs of nerves were used to compare the rate of Na^{22} uptake from a K-free solution with the rate of Na^{22} uptake from a K-free solution that contained 5 mM NaN_3. The nerves were soaked for 4 hours in their respective solutions, and then the rates of Na^{22} uptake were measured. The amount of Na^{22} taken up in 0.5 hour was lower in those nerves that had been exposed to the NaN_3 (35 μmols/gm *vs.* 43 μmols/gm). If this represented an effect of NaN_3 on passive permeability, it should also be that the nerves in NaN_3 contained less Na than their mates. However, this was not the case, for those nerves that had soaked in NaN_3 contained much more Na than did their mates (118 μmols/gm *vs.* 85 μmols/gm). This indicates that in the K-free solution the NaN_3 inhibited the Na efflux to a greater extent than it did the Na influx, and it shows that the primary effect of NaN_3 on the Na efflux is not due to an effect on passive permeability, but to an effect on active transport.

One other consideration should be mentioned. In the experiments that were reported here the net movements of Cl were small. Although Cl is not the only anion in nerve, it is the major anion in the bathing solution. Therefore, it is not unreasonable to assume that net movements of anions do not play a role in most of the Na-K exchanges that have been observed. (Obviously, this assumption is not true when a nerve is bathed in an isotonic solution of sucrose.) If this assumption is valid for solutions of ionic strength comparable to that of standard Ringer's solution, then, because of the requirement for electroneutrality, the net movements of Na and K must be equal in magnitude and opposite in direction. Therefore, the fact that Na and K are usually observed to exchange on an equimolar basis may result from a low anion permeability of the nerve membrane and from the requirement for electroneutrality, and not be due to the presence of a tightly coupled Na-K exchange pump.

APPENDIX

Consider an infinite plane sheet of thickness $2l$ that contains a substance, S, with a diffusion coefficient, D. The sheet is immersed suddenly in an infinite and well

stirred medium that contains no S. The rate at which S crosses the boundary between the sheet and the medium is proportional to the concentration of S at the surface of the sheet, the proportionality constant being α. The boundary conditions on the diffusion equation are:

$$\mp D \frac{\partial c}{\partial x} = \alpha c \text{ at } x = \pm l \qquad \text{(reference 2, p. 34)}$$

If the initial distribution of S within the sheet were uniform, then the amount of S remaining within the sheet at any time, t, is given by the equation:

$$S(t) = S(0) \sum_{n=1}^{\infty} \frac{2L^2 \exp(-\beta_n^2 Dt/l^2)}{\beta_n^2 (\beta_n^2 + L^2 + L)} \qquad (4)$$

where $L = l\alpha/D$ and β_n is a positive root of the equation $\beta \tan \beta = L$ (2, p. 57).

In the case of the diffusion of Na^{22} in a single fiber with an impermeable myelin sheath, the boundary conditions are:

$$\mp D \frac{\partial c_i^*}{\partial x} = \frac{c_i^*}{c_i} M_o' \quad \text{at} \quad x = \pm l$$

where M_o' = the Na efflux per unit area
c_i^* = axoplasmic concentration of Na^{22} in CPM/ml
c_i = axoplasmic concentration of Na in μmols/ml.

The efflux per unit area, M_o', is related to M_o, the efflux per gram dry, by the equation:

$$M_o' = M_o(V/A)(W/V)$$

where A = the total effective area for efflux at the two ends of the internode and is assumed to be equal to two times the cross-sectional area of the axis cylinder.
V = the volume of the axoplasm in the internode.
W = the dry weight of an internode.

The ratio of the volume of a cylinder of length $2l$ to the area of its ends is:

$$V/A = l$$

Hence, $M_o' = lM_oW/V$.

The concentration of radioisotope c_i^*, referred to a unit volume of axoplasm, is related to the concentration, $c_i^{*\prime}$, referred to the dry weight by the relation:

$$c_i^* = c_i^{*\prime} W/V$$

Similarly, for the stable isotope:

$$c_i = c_i' W/V$$

If the distribution of Na within the fiber is uniform and is in a steady state, then $c_i' = Q_i$, the measured intracellular Na content in μmols/gm.

Substituting these relations into the equation for the boundary conditions, one obtains:

$$\mp D \frac{\partial c_i^{*\prime}}{\partial x} = \frac{l M_o}{Q_i} c_i^{*\prime}$$

Therefore, α is analogous to lM_o/Q_i, and if the initial distribution of Na^{22} were uniform, then at any time, the quantity of Na^{22} remaining in the fiber would be given by Equation, 4, where:

$$L = l^2 M_o / Q_i D$$

The rate constant of the slowest mode of relaxation ($n = 1$) is

$$k_o = \beta_1^2 D / l^2$$

where β_1 is the first root of the equation $\beta \tan \beta = L$. This rate constant should be equivalent to the rate coefficient observed in the efflux experiments. For a given value of D, the rate constant is proportional to β_1^2, and β_1 is determined by the ratio of M_o to D. The question is: Are there values of D such that M_o may change appreciably without causing a significant change in β_1? To determine this, a value of L was assumed and β_1 was determined from Table 4.2, p. 329 of Crank (2). A value of D was calculated from the equation:

$$D = l^2 k_o / \beta_1^2$$

with $l = 1$ mm, and $k_o = 60$ per cent/hr., the experimental value. Then M_o was calculated from the equation:

$$M_o = L Q_i D / l^2 = L Q_i k_o / \beta_1^2$$

with $Q_i = 37$ μmols/gm dry.
Note that when L is small, β is small, and $\beta \tan \beta \approx \beta^2 = L$.
Therefore, for small L:

$$k_o = M_o / Q_i,$$

the value predicted by simple theory.

Neglecting internodal diffusion, the value of M_o is:

$$M_o = k_o Q_i = 0.6 \times 37 = 22 \ \mu \text{ mols/(gm} \times \text{hr.)}$$

Table II shows the values of L, β_1^2, D/D_o (the diffusion coefficient relative to its value in aqueous solution), and M_o that were obtained by taking into account internodal diffusion. The smallest value of L that has physical significance is somewhat greater than 0.1, corresponding to a value of D/D_o equal to 1. The values of the Na efflux do not differ greatly from the simple estimate until $L = 1.0$ or $D/D_o = 0.18$. If D/D_o were 0.18 and the Na efflux, M_o, were reduced suddenly by 50 per cent

TABLE II

VALUES OF L, β_1^2, D (THE CALCULATED VALUE OF THE DIFFUSION COEFFICIENT FOR Na IN THE INTERNODE), AND M_o, THE CALCULATED SODIUM EFFLUX

L	β_1^2	D/D_o*	M_o
			$\mu mols/(g \times hr.)$
0.1	0.097	1.40	22.9
0.2	0.188	0.71	23.5
0.3	0.273	0.50	24.4
0.5	0.426	0.31	26.1
1.0	0.74	0.18	30.0
2.0	1.16	0.12	38.1
5.0	1.73	0.08	64.1
10.0	2.05	0.06	109.
∞	2.46	0.05	∞

* The diffusion coefficient is expressed relative to D_o, the value in aqueous solution, which was assumed to be 1.3×10^{-5} cm²/sec. Further explanation in the text.

(by the action of a K-free solution, for instance), then L should fall from a value of 1.0 to 0.5 and reduce β_1^2 from 0.74 to 0.43, a reduction of 42 per cent. The fractional change in k_o would be 80 per cent of the fractional change in M_o, a relatively small error. Thus, it seems that if a K-free solution were to reduce the Na efflux from frog nerve, then this reduction would not be masked by the slowness of internodal diffusion, unless the mobility of Na in the axoplasm were less than 18 per cent of the mobility of Na in aqueous solution.

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